

Retinoic acid induces BDNF responsiveness of sympathetic neurons by alteration of Trk neurotrophin receptor expression

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Received 26 September 1994; revised version received 27 October 1994

Abstract The expression of high affinity neurotrophin receptors (TrkA, TrkB, and TrkC) determines the survival response of different populations of neurons to specific members of the neurotrophin family, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3). However, the mechanism which controls the expression of neurotrophin receptors during neuronal development is largely unknown. Here we show that the treatment of the cultured sympathetic neurons from newborn rat superior cervical ganglia (SCG) with retinoic acid (RA), a derivative of vitamin A, suppressed the expression of *trkA* mRNA and induced the expression of *trkB* mRNA. Expression of the functional TrkB receptor was confirmed by the emergence of trophic dependence of these neurons on BDNF in the absence of NGF. Differential regulation of *trk* mRNAs by RA provides a possible model for the establishment of neurotrophin dependence of peripheral neurons.

Key words: Sympathetic neuron; Retinoic acid; Neurotrophin; Neurotrophin receptor; Reverse transcription-polymerase chain reaction

1. Introduction

The survival, maturation, and function of post-mitotic neurons are regulated by neurotrophic factors. The neurotrophins, the NGF family of neurotrophic factors, were the first identified and are a still expanding group of neurotrophic factors acting on various types of neurons at different developmental stages [1,2]. Recently, the gene family of the receptor tyrosine kinase, Trk (now also referred to as TrkA as the receptor for NGF, while TrkB and TrkC denote the main receptors for BDNF and NT-3, respectively) was identified as a signal-transducing as well as ligand-specifying component of the high affinity neurotrophin receptor [3–6]. Genes for the Trk family receptors encode both full-length, 130–145 kDa proteins and truncated variants lacking an intracellular tyrosine kinase domain. Although the roles of truncated forms of Trk receptors expressed in both neuronal and non-neuronal cells remain to be clarified, full-length Trk receptors are expressed only in neuronal cells and mediate the biological action of neurotrophins on these cells.

Analysis of the regulation of *trk* mRNA expression should allow us to assay environmental signals which may induce or alter neurotrophin responsiveness of various types of neurons during neural development [3,7]. Some reports describe neurotrophin-responsive cell lines as model systems for the regulation of neurotrophin receptor expression [8–10]. Primary cultures of adrenergic sympathetic neurons from superior cervical ganglia (SCG) provide another model system to study neuronal differentiation of the peripheral nervous system of neural crest origin. Recently it has become apparent that neural crest-derived cells, such as sensory and sympathetic neurons, undergo a developmental switch in trophic factor dependence from neuro-

trophins (BDNF, NT-3), produced by autocrine and/or paracrine mechanism, to neurotrophins (NGF, BDNF), produced by target tissue [11]. Retinoic acid (RA), which has been shown to induce neurotrophin dependence of immature sympathetic neurons and neural crest cells [12,13], may be involved in such a developmental switch. In the present study, we examined whether RA affects the expression of neurotrophin receptors and neurotrophin responsiveness of cultured sympathetic neurons. The results demonstrate that RA induces BDNF responsiveness of SCG neurons by the differential alteration of *trk* mRNA expression.

2. Materials and methods

2.1. Primary culture of SCG neurons

SCG were dissected from 2-day-old Wistar rats, and were sequentially digested with collagenase (Sigma; 3 mg/ml) for 30 min and trypsin (Difco; 2 mg/ml) for 30 min at 37°C. After centrifugal washing, cells were dissociated by trituration. A single cell suspension was plated onto 35 mm culture dishes precoated with poly-D-lysine (Sigma; 0.1 mg/ml) and laminin (Collaborative Research; 1 µg/cm²) to give a density of approximately 5 ganglia per dish. On the day following seeding, the culture medium was replenished and cytosine-β-D-arabino-furanoside (Sigma; 10 µM) was added for 3 days to prevent proliferation of non-neuronal cells. SCG neurons were cultured in the absence or presence of all-trans retinoic acid (RA; Sigma; 0.1 µM) in the basal culture medium supplemented with NGF (Promega; 40 ng/ml) for 6 days. The basal culture medium consisted of Ham's F-12 medium (Sigma) containing BSA (0.1 mg/ml), streptomycin (0.1 mg/ml), penicillin (50 U/ml) and N2 supplement [14].

2.2. RNA preparation and Northern blot analysis

Total RNA was prepared from the cells according to the method of Chomczynski and Sacchi [15] with slight modifications as described previously [16]. For Northern blot analysis, total RNA (7 µg for *trkA* and *trkB*, 1.4 µg for p75) was subjected to electrophoresis through a 1.5% agarose gel and transferred to a nylon membrane (Hybond-N; Amersham). The membrane was hybridized with ³²P-labeled cRNA probe at 65°C in 50% formamide. A *trkA* cRNA probe corresponded to 618 bp of the extracellular domain of rat *trkA* cDNA [4], and a *trkB* cRNA probe was a mixture of 492 bp of the extracellular domain and 510 bp of the intracellular tyrosine kinase domain of rat *trkB* cDNA [5]. The p75 cRNA probe corresponded to 554 bp of the extracellular domain [17]. The membrane initially hybridized with the *trkA* probe

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Abbreviations: RA, retinoic acid; SCG, superior cervical ganglion; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; RT-PCR, reverse transcription-polymerase chain reaction.

was strip-washed and rehybridized with a 420 bp mouse β -actin cRNA probe to evaluate the level of β -actin expression as an internal control (cf. Fig. 1A).

2.3. RT-PCR

Aliquots (0.15 μ g) of the total RNA samples were treated with DNase I (Takara; 0.07 U/ μ l) at 37°C for 15 min, and single-strand cDNA was synthesized with 0.5 μ M random hexamer and AMV-XL reverse transcriptase (Life Sciences Inc.; 0.25 U/ μ l) at 42°C for 45 min. PCR was performed in a total volume of 50 μ l containing cDNA, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.1% Triton X-100, 0.5 μ M each 5' and 3' primers, 50 nCi/ml [α -³²P]dCTP and 0.02 U/ μ l Taq polymerase (Promega). Samples were subjected to 30–36 cycles of PCR according to the following scheme; 94°C for 1 min, 55°C for 1 min, 72°C for 2 min. The PCR products were electrophoresed on a polyacrylamide gel and the radioactivity incorporated into cDNA fragments was quantified with a Bio-Image Analyzer (Fuji). The amplified regions of the cDNA were as follows; *trkA*, 291–908[4]; *trkB(e)*, 116–607; *trkB(k)*, 1422–1931[5]; *trkC(e)*, 186–739; *trkC(k)*, 1459–1943[6]; *p75*, 160–713 [17,18]. Numbers denote nucleotide positions in the corresponding cDNA in the original references. Primer pairs for *trkA*, *trkB(e)* and *trkC(e)* and *p75* were located in the extracellular domain, while primer pairs for *trkB(k)* and *trkC(k)* were located in the intracellular tyrosine kinase domain.

2.4. Bioassay of neurotrophin activity

Cultures were established as described above. Dissociated SCG neurons were seeded into each 16-mm well of a 12-well plate at a density of 1.5 ganglia per well. During the period of the pretreatment, the 4 days following seeding, SCG neurons were cultured in the basal culture medium supplemented with NGF (40 ng/ml) alone or NGF + RA (0.1 μ M). The medium then was replenished and different neurotrophins (40 ng/ml) and RA (0.1 μ M) were added as indicated in Figs. 2 and 3. Wells devoid of added NGF received anti-mouse-NGF (Wako Chemicals, 5 μ g/ml) to neutralize residual NGF. At appropriate stages of culture, the number of surviving neurons (cells with round and phase-bright soma and neurites) within a 2 \times 3 mm rectangle in each well was counted. Human recombinant BDNF and human recombinant NT-3 were provided by Regeneron Pharmaceuticals Inc.

3. Results and discussion

3.1. Effects of RA on the expression of neurotrophin receptors in cultured SCG neurons

Dissociated sympathetic neurons require NGF but not BDNF for their survival in culture [19]. We also confirmed that in the presence of 40 ng/ml NGF, 83% of the plated SCG neurons dissociated from neonatal rat SCG had survived after 4 days in culture. In the presence of NGF, SCG neurons formed extensive neurite networks and could be cultured for more than 3 weeks without a significant decrease in cell number (see Fig. 3a). Accordingly the neonatal SCG neurons express high level of TrkA as well as p75, a low affinity common neurotrophin receptor which lacks an identifiable signal-transducing domain. In the absence of NGF or in the presence of BDNF alone, no neurons survived after 4 days in culture (also see Fig. 3g and c). In contrast, in the presence of 40 ng/ml NT-3, 19% of the plated neurons had survived after 4 days in culture (see also Fig. 3e), in agreement with reports that neonatal SCG neurons express a low level of NT-3 receptor (TrkC) compared to the high level of NGF receptor (TrkA) [20,21].

Total cellular RNA from neonatal SCG neurons cultured in the absence or presence of RA for 6 days was evaluated for changes in the levels of mRNAs for *trks* and *p75* by either Northern blot analysis or reverse transcription-polymerase chain reaction (RT-PCR). As shown in Fig. 1A, Northern blot analysis with total RNA from the cultured SCG neurons indicated that the RA treatment induced a remarkable increase in

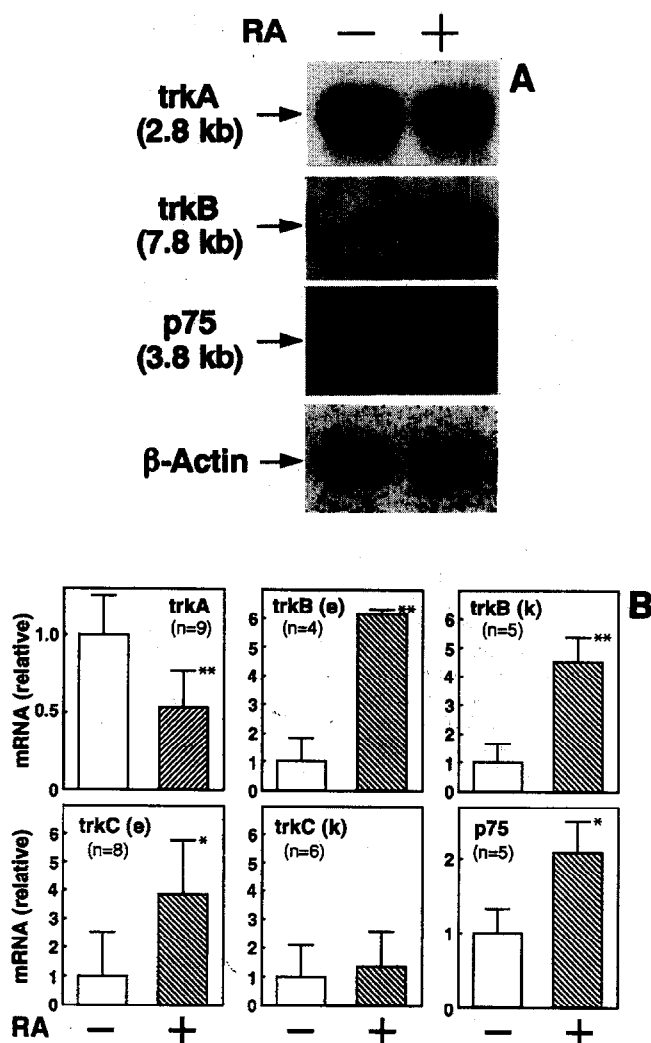


Fig. 1. RA differentially regulates the expression of neurotrophin receptor mRNAs in cultured sympathetic neurons. SCG neurons were cultured in the absence or presence of 0.1 μ M RA, in basal culture medium supplemented with NGF (Promega; 40 ng/ml) for 6 days. Then, total RNA was extracted from SCG neurons and subjected to (A) Northern blot analysis or (B) quantitative RT-PCR analysis for the evaluation of *trkA*, *trkB*, *trkC* and *p75* expressions. *P < 0.01; **P < 0.001; RA vs. control.

the level of 7.8 kb *trkB* mRNA species, which was almost undetectable in the untreated neurons. In the brain, the existence of multiple species of *trkB* mRNA encoding both the full-length TrkB receptor (4.8–9.0 kb), with intracellular tyrosine kinase catalytic domain, and the truncated form of TrkB (0.7–7.5 kb), lacking the tyrosine kinase domain, has been reported [5]. At present we cannot attribute the 7.8 kb *trkB* mRNA induced by RA in SCG neurons to a particular *trkB* mRNA species reported in the brain. To quantify the levels of *trkB* mRNAs derived from the extracellular domain and the intracellular tyrosine kinase domain, we designed two pairs of PCR primers corresponding to these two regions. RT-PCR experiments using these primers indicated low levels of expression of both the extracellular domain and the intracellular tyrosine kinase domain in the SCG neurons without the RA treatment. The RA treatment for 6 days resulted in increases in the expression of both the extracellular domain and the intracellular

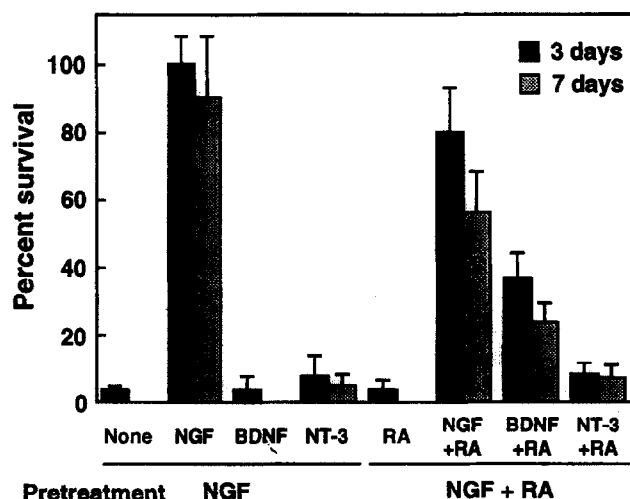


Fig. 2. RA pretreatment induces BDNF-dependent survival of cultured sympathetic neurons in the absence of NGF. SCG neurons were pretreated with NGF alone or NGF + 0.1 μ M RA for 4 days. Then, the medium was replenished and the SCG neurons were further cultured in the presence of indicated combinations of RA and neurotrophins. At the end of pretreatment and at 3 and 7 days after the pretreatment, the number of surviving neurons within rectangles in each well was counted (see section 2). Results were indicated as percentages of the number of neurons surviving in the same rectangle at the end of the pretreatment. Each bar represents the mean + S.D. from eight rectangles from two identical cultures.

lar tyrosine kinase domain by 6- and 4.5-fold, respectively (Fig. 1B, *trkB*(e), *trkB*(k)). These results strongly suggest that the RA treatment induced the expression of the full-length *TrkB* receptor with tyrosine kinase domain in the SCG neurons, although a concomitant increase in the expression of the truncated form of the *trkB* receptor can not be excluded.

In contrast, the RA treatment of the SCG neurons for 6 days led to suppression of the 2.8 kb *trkA* mRNA to 60% of the untreated level in Northern blot analysis while the expression of β -actin mRNA was unchanged (Fig. 1A). The RT-PCR experiment using a primer pair located in the extracellular domain of *trkA* cDNA also indicated that the RA treatment resulted in the suppression of the *trkA* mRNA to 50% (Fig. 1B, *trkA*). It is already known that neonatal rat SCG neurons express a high level of *trkA* mRNA with the size of 3.2 kb [21]. To our knowledge, the existence of any truncated form lacking the tyrosine kinase domain has not been reported for *trkA* mRNA. Therefore, our results strongly suggest that the RA treatment suppresses the expression of the functional *TrkA* receptor and leads to a reduction in the sensitivity of the SCG neurons to the NGF required for their survival.

Our RT-PCR experiment, however, detected certain levels of expression of both the extracellular domain and the intracellular tyrosine kinase domain of *trkC* mRNA in the SCG neurons without the RA treatment, corresponding to the NT-3 dependence of these neurons (Fig. 1B, *trkC*(e) and *trkC*(k), also see Figs. 2 and 3). The RA treatment selectively increased the

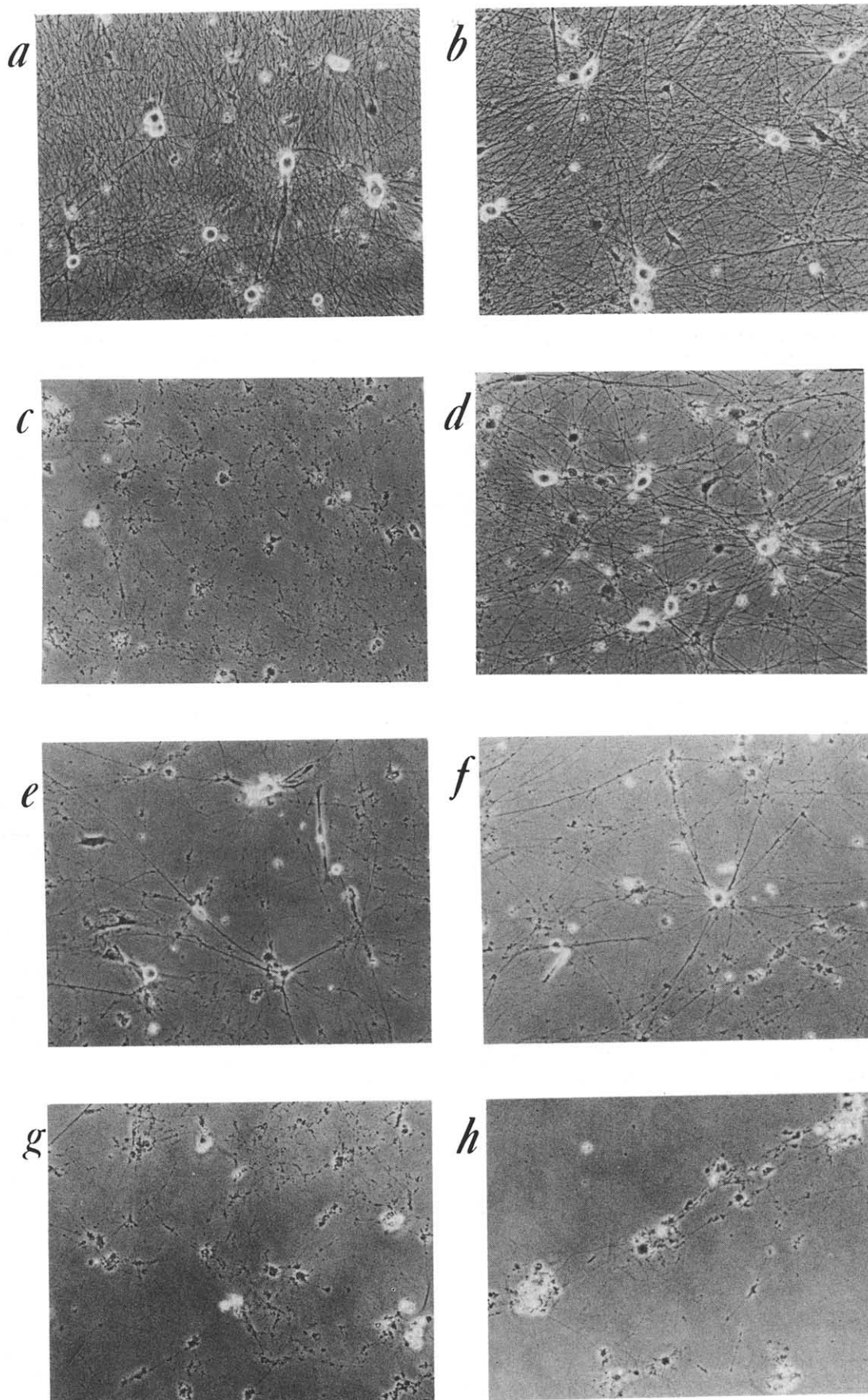
expression of the extracellular domain of the *trkC* mRNA by 4-fold, but not that of intracellular tyrosine kinase domain (Fig. 1B, *trkC*(e), *trkC*(k)). In addition, we found that the RA treatment increased the expression of p75 mRNA in the SCG neurons (3.5- and 2-fold as measured by Northern blot analysis and RT-PCR, respectively).

3.2. Induction of BDNF-dependent survival of SCG neurons by RA

We examined how the differential regulation of neurotrophin receptor expression by RA leads to the alteration of the specificity of neurotrophin responsiveness of the SCG neurons (Fig. 2). SCG neurons were cultured in the presence of NGF or NGF + 0.1 μ M RA for 4 days from the time of plating (the pretreatment period). The culture medium was then replenished and SCG neurons received different neurotrophins and anti-NGF antibody to neutralize the residual activity of NGF. After the pretreatment with RA + NGF, 37 and 23% of original number of SCG neurons found live at the end of the pretreatment survived at 3 and 7 days, respectively, in subsequent culture with BDNF and RA. However, after the pretreatment with NGF alone, no significant survival of SCG neurons was observed in subsequent culture with BDNF (Fig. 3c). Furthermore, the RA-pretreatment itself had no survival promoting effect on SCG neurons in subsequent culture in the absence of neurotrophins. As shown in Fig. 3d, SCG neurons surviving in the presence of BDNF after the RA pretreatment had a large, round cell soma and extended a fine network of neurites; but the neurite network was somewhat sparser than the that of SCG neurons cultured in the presence of NGF, corresponding to the decreased cell number in the presence of RA. These results clearly indicate that the survival responsiveness to BDNF was induced in a subpopulation of postnatal SCG neurons during the pretreatment period by RA, corresponding to the induction of *trkB* mRNA containing the tyrosine kinase domain. We do not know exactly how long it takes for RA to induce responsiveness to BDNF in the SCG neurons. We observed that the 3-day pretreatment with RA resulted in a survival response of SCG neurons to BDNF with a similar percentage of surviving neurons to that in the 4-day RA pretreatment. Therefore, we reasoned that 3 days is enough for RA to induce the expression of the functional receptor for BDNF. Furthermore, it is suggested that the maximum size of a subpopulation of SCG neurons which expresses the functional BDNF receptor at levels above threshold to give a survival response to BDNF is one-third of the original population.

Although, in the culture medium containing NGF without RA, SCG neurons survived for more than 3 weeks without a significant decrease in cell number, the pretreatment of SCG neurons with RA induced a gradual decrease in cell number even in the presence of NGF (Fig. 2, bar RA + NGF). Since the RA pretreatment suppressed the expression of the *trkA* mRNA, it is reasonable to conclude that RA suppresses the NGF responsiveness of the SCG neurons. Additionally, SCG neurons cultured in the presence of RA also formed an exten-

Fig. 3. Phase-contrast photomicrographs of sympathetic neurons in culture with different neurotrophins and RA. Cultures of SCG neurons were established as described in section 2. After the pretreatment with NGF alone (a, c, e, g) or NGF + 0.1 μ M RA (b, d, f, h) for 4 days, the medium was replenished and the SCG neurons were further cultured in the presence of the following combinations of RA and neurotrophins for 7 days before photographs were taken. (a) NGF; (b) RA + NGF; (c) BDNF; (d) RA + BDNF; (e) NT-3; (f) RA + NT-3; (g) none; (h) RA. Bar = 100 μ m.



sive network of neurites, although these SCG neurons tended to extend thicker bundles of neurites compared with the SCG neurons cultured in the absence of RA (Fig. 3b).

In contrast, the inclusion of RA during the pretreatment period did not significantly change the number of surviving neurons in culture medium containing NT-3. However, subsequent to the 4-day pretreatment period, percentages of SCG neurons that survived in the presence of NT-3 for 3 days (pretreatment with NGF, 7.2%; pretreatment with NGF + RA, 8.0%) were much smaller than those of SCG neurons surviving at 4 days with NT-3 from the time of plating (NT-3, 19%; NT-3 + RA, 14%). This result coincides with the progressive loss of the *trkC* receptor in SCG neurons during the late embryonic and neonatal period [20,21], which seems to be a process independent of the action of RA.

Differential regulation of the expression of *trk* neurotrophin receptors by RA has profound implications on the establishment of neurotrophin responsiveness of various types of neurons during development. Induction of *trkB* mRNA by RA is also reported in some human neuroblastoma cell lines [9]. However, regulation of *trkA* mRNA by RA is not known in these cell lines. Recently, it has become apparent that sympathetic neurons undergo a developmental switch in trophic factor dependence [11]. At the embryonic stage, NT-3 promotes survival of sympathetic neuroblasts via activation of TrkC [20,21]. As sympathetic neurons start innervating their targets during late embryonic and neonatal stages, the expression of TrkC is replaced by onset of the expression of TrkA. Hence, they become dependent on NGF produced by the target tissue [20,21]. We do not know whether RA affects TrkC expression and NT-3 responsiveness of sympathetic neurons during embryonic development, rather, we would like to include BDNF/TrkB as another neurotrophin/receptor system which supports survival and maturation of sympathetic neurons at their early developmental stage. RA has been shown to promote the survival and proliferation of neurogenic precursors in cultured neural crest cell populations [13]. Subpopulations of neural crest-derived cells in dorsal root ganglia and sympathetic ganglia in the chick have been found to express a certain type of nuclear RA receptor [22]. These observations suggest that RA acts on the early stage of the differentiation of neural crest-derived neurons such as sensory and sympathetic neurons. Recently, it was reported that RA induces high affinity NGF receptor and NGF dependence in chick sympathetic neuroblasts [12]. However, in our cultured rat sympathetic neuron system, RA suppressed the expression of *trkA* mRNA. This finding coincides with the observation that RA did not induce *trkA* mRNA in MAH cells, immortalized rat sympathoadrenal progenitor cells, where depolarization is a major TrkA-inducing signal [8]. Rather, induction of high affinity NGF receptor and NGF dependence in the chick system might reflect the increase of p75 expression by RA [10].

In a separate study, we showed that unmyelinated Schwann cells in a premature state are capable of synthesizing and secreting BDNF [23]. Interestingly, expressions of BDNF and NGF mRNA are differentially regulated in Schwann cells. Furthermore, it has been shown that ciliary neurotrophic factor (CNTF) induces responsiveness of neonatal rat SCG neurons to trophic factor(s) (which could be BDNF) produced by Schwann cells [24]. CNTF resembles RA in its action on SCG neurons. Thus, both CNTF and RA induce cholinergic differ-

entiation in several cell types, including neonatal rat SCG neurons [16,25]. Therefore, it is highly plausible that during early stages of development of SCG neurons, RA and other factors induce TrkB expression and responsiveness to BDNF produced by ganglionic non-neuronal cells such as Schwann cells. The trophic functions of the NT-3/TrkC and BDNF/TrkB systems in sympathetic neurons at the embryonic stage could be complementary or compensate each other, since the disruption of any single gene among the four neurotrophin/receptor genes does not lead to complete loss of sympathetic ganglia [26–31]. To further confirm the role of RA in the development of sympathetic neurons it is necessary to follow the developmental changes of the supply of RA within or in the periphery of the sympathetic ganglia and the expression of the relevant retinoid receptors. It is also intriguing to examine the effects of RA on neurotrophin responsiveness in other systems, such as sensory and spinal motor neurons, where a neurotrophic function of RA has been suggested [32,33]. In conclusion this is the first report that a sympathetic neuron can become dependent on BDNF by the RA-induced differential regulation of neurotrophin receptors, which would serve as a model system for the establishment of neurotrophin responsiveness.

Acknowledgements: We are grateful to Regeneron Pharmaceuticals Inc. for the supply of BDNF and NT-3. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (04268103) from the Ministry of Education, Science and Culture, Japan. M.K. is a recipient of a fellowship of the Japan Society for the Promotion of Science for Japanese Junior Scientists.

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